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Mosaic Eyes is a novel component of the Crumbs complex and negatively regulates photoreceptor apical size

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Establishment of apical-basal cell polarity has emerged as an important process during development, and the Crumbs complex is a major component of this process in *Drosophila*. By comparison, little is known about the role of Crumbs (Crb) proteins in vertebrate development. We show that the FERM protein Mosaic Eyes (Moe) is a novel regulatory component of the Crumbs complex. Moe co-immunoprecipitates with Ome/Crb2a and Nok (Pals1) from adult eye and in vitro interaction experiments suggest these interactions are direct. Morpholino knockdown of *ome/crb2a* phenocopies the *moe* mutations. Moe and Crumbs proteins colocalize apically and this apical localization requires reciprocal protein function. By performing genetic mosaic analyses, we show that *moe*$^{-}$ rod photoreceptors have greatly expanded apical structures, suggesting that Moe is a negative regulator of Crumbs protein function in photoreceptors. We propose that Moe is a crucial regulator of Crumbs protein cell-surface abundance and localization in embryos.

KEY WORDS: Inner segment, Outer segment, Renewal, Morphogenesis, Zebrafish

INTRODUCTION

Drosophila Crumbs (Crb) and vertebrate Crumbs orthologs are important regulators of epithelial morphology and apical polarity (Wodarz et al., 1995; Tepass et al., 1990; Roh et al., 2003) and are important for normal photoreceptor morphogenesis and zonula adherens junction formation and/or maintenance in *Drosophila* (Pellikka et al., 2002; Izaddoost et al., 2002; Mehalow et al., 2003; van de Pavert et al., 2004). Mutations in human Crumbs Homolog 1 (CRB1) are associated with the inherited retinal diseases retinitis pigmentosa 12 and Leber's congenital amaurosis (LCA) and retinal lamination defects have been observed in LCA (den Hollander et al., 1999; den Hollander et al., 2001; Lotery et al., 2001; Gerber et al., 2002; Jacobson et al., 2003). Deficiencies in mouse Crb1 are also associated with retinal abnormalities (Mehalow et al., 2003; van de Pavert et al., 2004), but these are less severe than those observed in humans, and photoreceptor development appears largely normal under normal light conditions. Loss-of-*crb* function in Drosophila photoreceptors causes defects in zonula adherens formation, stalk length and rhabdomere morphology, but polarity appears normal (Izaddoost et al., 2002; Pellikka et al., 2002).

The small intracellular domains of Crumbs and vertebrate Crumbs orthologs are highly conserved and encode two important protein interaction domains, a predicted FERM- (Band 4.1, Ezrin, Radixin, Moesin) binding domain and a PDZ-binding domain (Wodarz et al., 1995; den Hollander et al., 2000; Izaddoost et al., 2002; Roh et al., 2003). The orthologous Maguk proteins, Drosophila Stardust and mammalian Pals1, have been shown to bind to the PDZ-binding domain in *Drosophila* Crb and mammalian CRB1 and CRB3 (Bachmann et al., 2001; Hong et al., 2001; Kantardzhieva et al., 2005; Roh et al., 2002). The FERM protein, *D*Moesin, has been shown to be in a complex with Crb and shows some subcellular overlap with Crb in embryonic epithelia (Medina et al., 2002), but a direct interaction has not been demonstrated.

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We previously reported that zebrafish *mosaic eyes* (*moe*), which encodes a FERM protein, is required for normal retinal lamination and suggested that it might interact with the predicted FERM-binding domain in Crumbs proteins (Jensen et al., 2001; Jensen and Westerfield, 2004). The phenotype of *moe* mutants is similar to the phenotype of mutants in *nagie oko* (*nok*) (*mpp5* – Zebrafish Information Network), which encodes Pals1 (Wei and Malicki, 2002), and mutants deficient in both loci are indistinguishable from the single mutants, suggesting a genetic interaction between the two genes (Jensen and Westerfield, 2004). In this study we show that Moe interacts directly with Crb proteins and Nok (Pals1), and also forms a complex with Has (aPKCλ; Prkci – Zebrafish Information Network). Morpholino knockdown of one of the zebrafish Crumbs orthologs, *crb2a,* phenocopies the *moe* mutations. Finally, we show that *moe* is required for normal photoreceptor morphology; the apical domain is expanded in rod photoreceptors that lack *moe* function, suggesting that Moe may negatively regulate Crumbs protein function.

MATERIALS AND METHODS

Animals

AB wild-type strain, *nok*^{m520}, *has*^{m567} *moe*^{b476}, *moe*^{b781} and *moe*^{b882} alleles were maintained and staged as previously described (Jensen et al., 2001). The *moe*^{b476} and *moe*^{b781} alleles were crossed into the Rhodopsin-GFP (Xop-GFP) transgenic line (Fadool, 2003).

UV-opsin GFP transgenic fish construction

UV-opsin GFP transgenic fish were generated by cloning a 7 kb *Sac*I fragment from a UV-opsin^{+} PAC clone, including ~870 bases of the coding region plus 6 kb upstream, into the pG1/pESG GFP vector to generate a fusion protein that includes most of the UV opsin protein with GFP at the C-terminus. Linearized plasmid (60 ng/μl) was injected into single-cell embryos; germline founders were identified by PCR.

In situ hybridization

Whole-mount and section in situ hybridizations were performed as previously described (Jensen and Westerfield, 2004). cDNAs were linearized and transcribed: *crb2b Xho*I, SP6; *crb2a Not*I, SP6; mouse *ymo1 Bgl*II, T3; mouse *crb1* (Accession number BM941539) *Not*1, T3; mouse *crb2* (Accession number BI738283) *Eco*RI, T7.

Morpholino knockdown

crb2a splice-blocking morpholinos were targeted to the intron (~810 bases) between the second and third coding exons. Donor morpholino sequence was TTGCACTTCAATTACCTGTATATCC and acceptor was ACAGT-

TTACACCTACAGAGATCACA. Injection of donor morpholino (200 μmol/l) produced no discernable abnormality, while injection of acceptor morpholino (200 μmol/l) produced a weak *moe*-like phenotype in about 50% of injected embryos. Co-injection of both morpholinos (each 200 μmol/l) produced a *moe*-like phenotype in all injected wild-type embryos. Morpholino activity was tested by RT-PCR on RNA isolated from 60 30-hpf injected embryos. Primers used for RT-PCR were: RT, GCGGTC-GTGGCAAAGTC); PCR, GGCGAGACCTGTGAAGAAGACC and CCGTTTTGACAGGGATTTGACTC. Co-injection of *nok* splice-blocking morpholinos (each 200 μmol/l; donor, GTTTATGACAC-CCACCTAGTAAAGC; acceptor, CTCCAGCTCTGAAAGTACAA-ACACA) produced a phenotype indistinguishable from *nok* mutants (not shown).

Construction and expression of fusion proteins

GST-tagged Crb^{intra} proteins: sequences encoding the intracellular domains of Crb proteins plus variable amounts of transmembrane domain were cloned into pGEX-4T-1 (Amersham); Crb1, Crb2a, Crb2b, Crb3a and Crb3b are 40, 42, 42, 61 and 65 amino acids, respectively. A Moe fragment (EST accession number CD750925) encoding amino acids 1-434 was cloned into pRSET-A to make His-Moe_FERM. For MBP Moe_FERM and MBP-Moe_C-terminus sequence encoding residues 59-383 and sequence encoding residues 383-772, respectively, was cloned into pMal C2X (NEB). To make His-Nok proteins, full-length Nok and a fragment encoding the first 411 amino acids (Nok-N, including the PDZ domain) were cloned into pRSET-A or -B (Invitrogen). To make GST-Nok-Int, a 468 bp internal *Stu*I-*Pst*I fragment of Nok encoding the predicted Band 4.1 interacting region was cloned into pGEX4T-1.

Antibody production

Polyclonal antibodies to Moe were generated by immunizing rabbits (UMASS antibody facility) and guinea pigs (Rockland Immunochemicals) with purified His-Moe_C term fusion protein (amino acids 383-772, Accession number CD750925).

Biochemical assays

Co-immunoprecipitation

About 190 adult zebrafish eyes were homogenized with 5 ml cold IP lysis buffer, incubated for 1 hour at 4°C, centrifuged, and supernatant collected. For each reaction, 500 μl lysate was pre-cleared with 20 μl normal rabbit serum and 50 μl protein A/G Plus-Agarose (Santa Cruz Biotech). Pre-cleared lysate was collected by centrifugation and incubated with 20 μl normal rabbit serum or 10 μl anti-CRB3 antibodies at 4°C overnight. Twenty-five microliters of protein A/G Plus-Agarose and 300 μg BSA were added subsequently to capture the immunocomplex and incubated for 2 hours at 4°C. Resin was washed six times with lysis buffer. Co-immunoprecipitated proteins were eluted with reducing sample buffer and analyzed by western blotting.

Western blot analysis

Western blot analysis was performed on whole embryo or larval lysates, affinity-purified proteins or purified fusion proteins. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane, blocked in blotto, incubated overnight in primary antibody at 4°C; anti-CRB3 (1:1500), anti-GST-HRP (1:9000-1:20000; Amersham), rabbit anti-Moe (1:1500), guinea pig anti-Moe (1:750), anti-Nok (1:2500), anti-PKCζ (1:1000; Santa Cruz Biotech, C-20), mouse anti-α-tubulin (1:2000; 12G10 DSHB), washed, incubated in anti-rabbit-HRP (1:70000-100000; Pierce), anti-guinea pig-HRP (1:15000, Jackson), or anti-mouse-HRP (1:20000; Pierce), washed, detected with SuperSignal West Dura (Pierce) or West Pico (Pierce) substrate and exposed to X-ray film.

Far western

One microgram of bait fusion protein was resolved on SDS-PAGE, transferred to nitrocellulose, blocked with blotto and incubated with interacting fusion protein (2.5 μg/ml) in 3% milk/PBSTw at 4°C overnight. Bound fusion protein was detected by using anti-GST or anti-MBP (1:10000; NEB) or anti-Omni (1:1000; Santa Cruz Biotech). Immunoreactivity was revealed as above.

Pulldown assays

About 1000 3d zebrafish were homogenized in 1 ml cold lysis buffer plus protease inhibitors, centrifuged, supernatant isolated, and TritonX-100 added to 1%. One milligram of purified His-Moe_FERM or His-*lacZ* was immobilized on Ni-NTA resin (Qiagen), washed, incubated with 1 ml lysate for 1 hour at 4°C, resin was washed with 10 ml HEPES column buffer (with and without 1% TritonX-100) and proteins eluted with reducing sample buffer, 20-30 μl eluant (total eluant volume, 700 μl), resolved by SDS-PAGE, 0.5-1.5% of the lysate was included on the gel and analyzed by western blot. In parallel, 500 3d Nok morpholino-injected zebrafish were homogenized and 200 μg His-Moe_FERM was used for pulldown as described above. Five hundred microliters of adult eye lysate was incubated with either 200 μg immobilized His-Moe_FERM or His-*lacZ* overnight at 4°C. Resin was washed and proteins eluted with 90 μl sample buffer.

In vitro GST pulldowns

For Moe-Crb protein interactions, 10 μg His-Moe_FERM was bound to NHS resin (Amersham). Residual reactive sites were blocked with 100 mmol/l ethanolamine. The resin was incubated with 10 μg GST-Crb1, GST-Crb2a or Crb2b for 1 hour at 4°C. Bound proteins were eluted with sample buffer. For Moe-Nok interactions, 10 μg GST or GST Nok-fusion proteins were bound to GST microspin columns (Amersham), washed and incubated with 20 μg BSA in PBS for 2 hours at 4°C, incubated overnight at 4°C with the GST-tagged protein in PBS (plus 20 μg BSA). Columns were washed, protein glutathione-eluted and analyzed by western blot with tag antibodies.

Immunocytochemistry and microscopy

Zebrafish were fixed in 4% PFA, and sections (18 or 30 μm) permeabilized with 0.1% SDS for 15 minutes, washed in PBSTw, and incubated in blocking solution then with primary antibody overnight at 4°C: rabbit anti-Moe 1:1000; guinea pig anti-Moe 1:500; anti-CRB3 1:400; anti-Nok 1:400; anti-aPKCζ 1:1000; mouse anti-ZO-1 1:10; rabbit anti-GFP 1:300 (Molecular Probes); mouse anti-Rhodopsin 1:100; mouse Zn5 1:5 (ZIRC); anti-phospho-H3 1:1000 (Upstate Biotech). Sections were washed, incubated with secondary antibody [-FITC/-TRITC (Molecular Probes) 1:200, -CY5 1:100 (Jackson)] and analyzed with a Zeiss LSM 510 Meta Confocal System. Confocal images are a single scan (four to eight averaged), optical thickness ~1 μm. Images in Fig. 2 were taken with the same settings.

Blastomere transplantations and generation of retina mosaics

Donor embryos were produced by incrossing Rhodopsin-GFP;$moe^{b781/+}$ fish. At the high stage to dome stage, ~20-40 cells were transplanted from labeled-donor embryos into wild-type host embryos (Jensen et al., 2001). Larvae were placed in the dark for 2 hours and were fixed and sectioned (30 μm) as described above. Confocal z-stack images (~1 μm optical thickness) were acquired every 0.38 μm. Cell volume was approximated by accumulating the area of the cell on each z-section: cell area was outlined and measured in each z-section and for each cell the area from the z-sections was summed to represent volume. Only cells completely captured in the z-stack were included in the calculations. Measurements were repeated and averaged to reduce sampling error. Donor cells were not lineage traced with rhodamine dextran for the experiments localizing panCrb and ZO-1 in GFP^+ rods so that rhodamine could be used for antibody localization.

RESULTS

Identification and expression of Zebrafish *crumbs* orthologs

There are three vertebrate Crumbs orthologs, Crb1, Crb2 and Crb3 (den Hollander et al., 1999; van den Hurk et al., 2005; Makarova et al., 2003). In order to test our hypothesis that Moe forms a complex with Crumbs proteins, we had to clone zebrafish *crumbs* genes. We used sequences from the zebrafish genome project to clone *crb1*, *crb2a*, *crb2b* and *crb3b*, and found an EST for *crb3a*. These are the same *crumbs* genes recently described by Omori and Malicki (Omori and Malicki, 2006). To determine whether any of these *crumbs* orthologs are coexpressed in the same cells and tissues as *moe*, we performed in situ hybridization in embryos and eyes. At 30

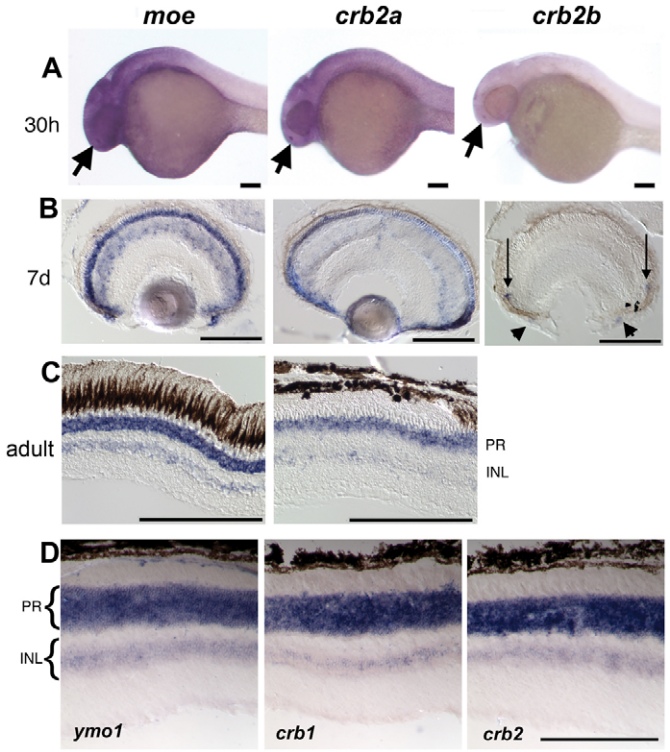

Fig. 1. Expression of zebrafish *crb2* genes. (**A**) *moe* and *crb2a* are expressed in the brain, eye and nose (arrows) at 30 hpf; *crb2b* is expressed in the nose (arrow) and weakly or not at all in the brain and eye. (**B**) *moe* and *crb2a* are expressed in photoreceptors, cells in the inner nuclear layer, progenitor cells in the periphery and cells in the anterior chamber at 7 dpf; *crb2b* expression is detected in a small number of newly differentiating photoreceptors near the ciliary margin (arrows) and in cells in the anterior chamber (arrowheads). (**C**) Zebrafish *moe* and *crb2a* are expressed in photoreceptors and cells in the inner nuclear layer in adults. (**D**) Mouse *ymo1*, *crb1* and *crb2* are expressed in photoreceptors and cells in the inner nuclear layer in the adult. Scale bars: 100 μm. INL, inner nuclear layer; PR, photoreceptors.

hours post-fertilization (hpf), *moe* and *crb2a* were expressed in the skin, nose and epiphysis, and ubiquitously in the brain and eye; *crb2b* was very weak in the brain but highly expressed in the epiphysis and nose (Fig. 1A). At 7 days post-fertilization (dpf), *moe* and *crb2a* were expressed in photoreceptors, cells in the inner nuclear layer, proliferating cells in the peripheral margin, and cells in the anterior chamber (Fig. 1B); *crb2b* was detected in a small number of newly differentiating photoreceptors in the periphery and cells in the anterior chamber (Fig. 1B). At 3 dpf, we observed high expression of *crb2b* in newly differentiating photoreceptors and no expression of *crb1, crb3a* or *crb3b* in the retina (data not shown) like that observed by Omori and Malicki (Omori and Malicki, 2006). In adults, *moe* and *crb2a* were expressed in photoreceptors and cells in the inner nuclear layer (Fig. 1C).

We also examined the expression of the mouse ortholog of *moe*, *ymo1* (*Drosophila yurt* and zebrafish *moe like 1*; annotated *Erythrocyte Protein Band 4.1 Like 5* in the human genome), along with *crb1* and *crb2* in the adult mouse retina. We found that *ymo1* was expressed in the same population of cells as *crb1* and *crb2* and was expressed in photoreceptors and a subset of cells in the inner nuclear layer (Fig. 1D), similar to that expression previously shown by den Hollander and colleagues (den Hollander et al., 2002) and van den Hurk and colleagues (van den Hurk et al., 2005), although we did not detect *crb2* expression in the retinal ganglion layer or in bipolar cells like that reported by van den Hurk and colleagues (van den Hurk et al., 2005). Coexpression in the same populations of cells of *moe* and *crb2a/b* genes in zebrafish and the orthologous genes in mouse is consistent with the idea that the genes/proteins interact.

Morpholino knockdown of *crb2a* phenocopies *moe* mutations

To test whether loss-of-function of zebrafish *crumbs* orthologs would phenocopy the *moe* mutations, we targeted knockdown of *crb2a* gene function, because its expression most closely resembles *moe* expression. Injection of *crb2a* splice-blocking morpholinos into one to two-cell wild-type embryos (hereafter called *crb2a* morphants) inhibited splicing as tested by RT-PCR (see Fig. S1A in the supplementary material) and produced a phenotype that is indistinguishable from *moe* mutants, including reduced brain ventricles, edema of the heart cavity and patchy retinal pigmented epithelium (see Fig. S1C,D in the supplementary material). The similarity in phenotype between *crb2a* morphants and *moe* mutants extended to loss of retinal lamination and the ectopic localization of retinal ganglion cells and rod photoreceptors (see Fig. S1E-P in the supplementary material). *crb2a* morphants died around 5-6 dpf, similar to *moe* mutants. The observation that *moe* and *crb2a* loss of function cause similar phenotypes further supports the idea that *moe* and *crumbs* genes/proteins interact. Omori and Malicki (Omori and Malicki, 2006) reported recently that the *ome* locus is *crb2a* and our *ome* morphant phenotype was like their *ome* morphants.

Localization of Moe and Crumbs proteins requires reciprocal protein function

If Moe forms a complex in vivo with Crb proteins, the proteins should show some colocalization. To examine the localization of Moe protein, we generated Moe polyclonal antibodies and showed by western blot that a protein of ~110 kDa was recognized by the antibody in wild type and was absent in all *moe* mutant alleles (Fig. 2A). The molecular weight of Moe protein is larger than predicted (~89 kDa), suggesting that it may be modified post-translationally. To examine the localization of Crumbs proteins, we determined by western blot that the antibody raised against the intracellular domain of human CRB3 (Makarova et al., 2003) recognized the recombinant intracellular domains of all the zebrafish Crumbs proteins we identified (Fig. 2B), and thus anti-CRB3 should be considered a pan-Crb antibody in zebrafish, and hereafter is referred to as anti-panCrb.

We examined the localization of Moe and panCrb in wild-type embryos and whether their localization depends on the function of *moe*, *nok* (*pals1*), *crb2a/ome* and *has* (*aPKCλ*). We included *has* mutants (Horne-Badovinac et al., 2001) because its phenotype is similar to *moe*, *ome* and *nok* mutants and *crb2a* morphants. In addition, the Par complex, which includes aPKCλ, interacts with Crumbs complexes (Wodarz et al., 2000; Hurd et al., 2003; Lemmers et al., 2004; Nam and Choi, 2003), and in *Drosophila*, Crumbs is a target of DaPKC (Sotillos et al., 2004). In 30 hpf wild-type embryos, both Moe and panCrb were concentrated at the apical surface of the brain and retinal neuroepithelium; Moe also localized cortically in all neuroepithelial cells (Fig. 2C,D). In *moe* mutants, Moe was lost, and apical panCrb in the brain and retinal neuroepithelium was lost (Fig. 2E,F). In *crb2a* morphants, *ome* and *nok* mutants, apical Moe and panCrb were lost, although in *nok* mutants disorganized plaques of Moe labeling were observed near the brain midline (Fig. 2G-J,M,N). In *has* mutants, apical Moe and panCrb in the brain and retinal neuroepithelium were reduced and patchy compared with

wild-type embryos (Fig. 2K,L). Double labeling and colocalization of Moe and panCrb is provided in Fig. S2 in the supplementary material. Both Moe and panCrb localize to the apical neuroepithelium and their localization is co-dependent, further supporting the idea that the proteins might interact.

Loss of panCrb labeling at the apical surface in *moe*, *nok* and *ome* mutants could be due to loss of Crb proteins; or, alternatively, Crb proteins may be present but no longer localized apically and instead are diffuse. To distinguish between these two possibilities, we performed western blot analysis of 30 hpf wild-type, *moe*, *nok* and *ome* mutants. In wild-type embryos, a protein of about 150 kDa was recognized by anti-panCrb antibody. The predicted molecular weight of Crb2a is about 156 kDa, and it is by far the most abundantly expressed *crumbs* gene at 30 hpf; thus this protein is probably Crb2a. Levels of this protein were unaffected in moe^{b781} mutants (and moe^{b476} deficiency, not shown), barely detectable in *crb2a* morphants and *nok* mutants, was absent in *ome* mutants (Fig. 2O) and moderately reduced in *has* mutants (data not shown). Expression of *crb2a* and *crb2b* mRNA by in situ hybridization was unaffected in *crb2a* morphants, *moe*, *nok* and *ome* mutants (data not shown). These results suggest that Moe is required either for apical localization (or retention) or for trafficking of Crumbs proteins.

Localization of Moe, Crumbs proteins, Nok and aPKCλ in the retina

Because Crumbs proteins are important for retinal development, we examined whether Moe colocalizes with panCrb in the retina as well as with Nok (Pals1) and Has (aPKCλ). At 7 dpf, Moe localized to the photoreceptor layer, retinal progenitors in the periphery and cells in the anterior chamber (Fig. 3A-F), while panCrb localized to the photoreceptor layer apical to the outer limiting membrane (OLM) and the apical surface of progenitor cells in the periphery (Fig. 3A). Merging the Moe and panCrb images shows that anti-Moe and anti-panCrb strongly colocalized in newly differentiating photoreceptors (Fig. 3A, Moe+anti-panCrb, bracket) at the periphery of the

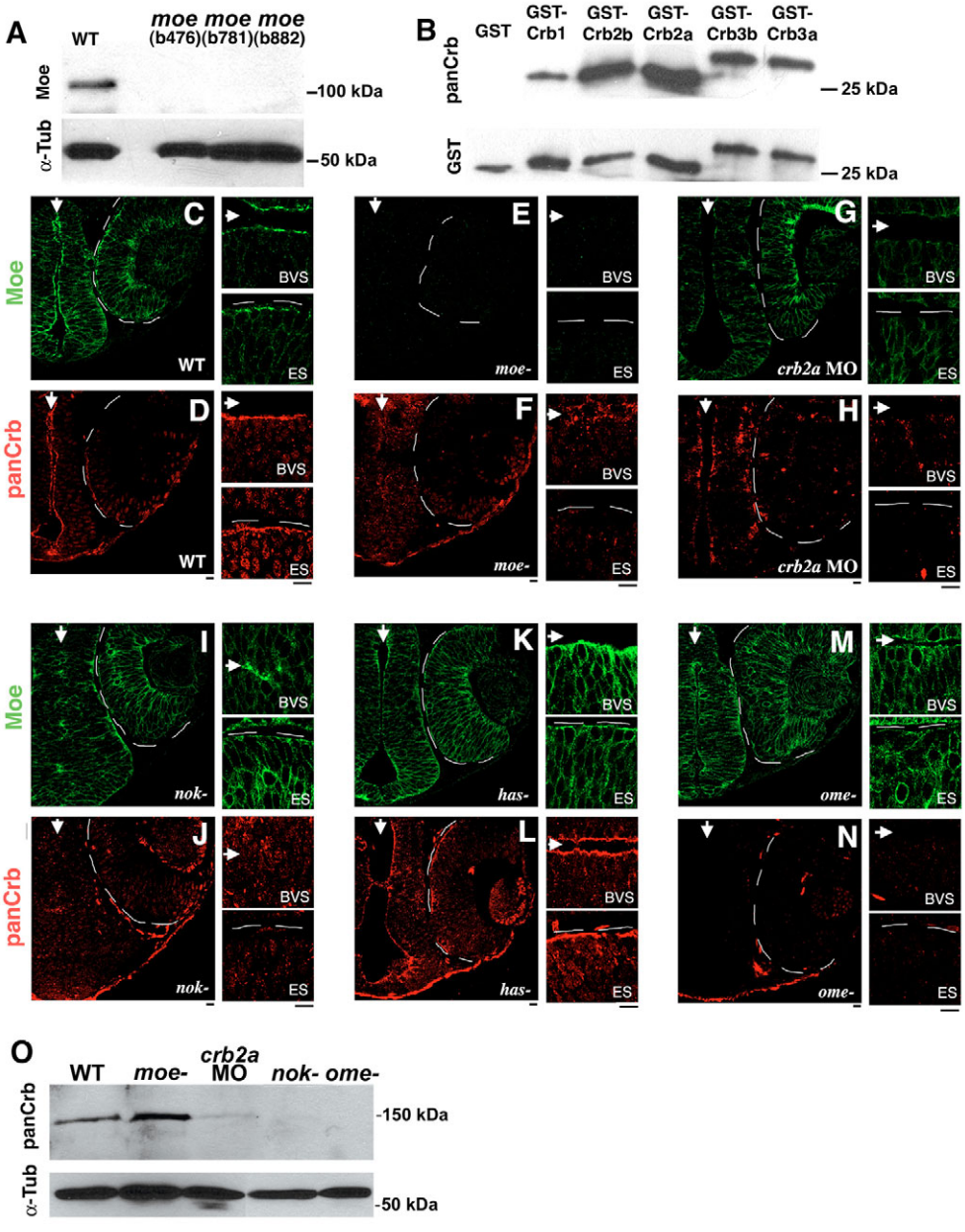

Fig. 2. Localization of Moe and Crumbs proteins in embryonic brain and eye. (**A**) Western blot of wild type, moe^{b476}, moe^{b781}, moe^{b882} 3d larvae probed with rabbit anti-Moe. Anti-Moe recognizes a protein of approximately 110 kDa in wild-type extracts that is absent in *moe* mutants. The blot was reprobed with anti-α-Tubulin as a loading control. (**B**) Anti-CRB3 recognizes the intracellular domains of all zebrafish Crumbs proteins. Western blot of GST, GST-Crb1^{intra}, GST-Crb2a^{intra}, GST-Crb2b^{intra}, GST-Crb3a^{intra}, GST-Crb3b^{intra} probed with anti-CRB3 antibodies. The blot was reprobed with anti-GST as a loading control. (**C-N**) Moe (green) and panCrb (red) labeling in 30 hpf brain and eye in wild-type (C,D), moe^{b781} (E,F), *crb2a* morphant (G,H), *nok* (I,J), *has* (K,L) and *ome* (M,N) embryos. Arrows indicate brain ventricles and eyes are outlined. High magnification of the apical ventricular surface of the brain (BVS) and eye/retinal surface (ES) are shown to the right. Scale bars: 10 μm. (**O**) Western blot of 30 hpf wild-type, moe^{b781}, *crb2a* morphant, *nok* and *ome* embryos probed with anti-panCrb antibodies. One-fifth of the material loaded for the panCrb Western was probed with anti-α-Tubulin to show relative loading amounts.

photoreceptor layer. Higher magnification of Moe and panCrb colocalization in the photoreceptor layer was shown at 4 dpf, a time when most photoreceptors are forming outer segments (Fig. 3B). Moe also colocalized with Nok and aPKCλ in the photoreceptor layer and with aPKCλ in the outer plexiform layer (Fig. 3C-F). Colocalization of Moe with panCrb, Nok and aPKCλ places these proteins in a position to potentially interact.

We also examined the localization of Moe and panCrb relative to markers for photoreceptors and Müller glia, which send processes into the photoreceptor layer. Moe and Crb proteins appeared to be in all photoreceptor types examined, and Moe was in Müller processes that project into the photoreceptor layer (Fig. 4A-G). We also examined Moe and panCrb localization in the photoreceptor region relative to the OLM, a specialized adherens junction between photoreceptor cells and Müller glia and between individual Müller cells and individual photoreceptors (Williams et al., 1990). In mouse retina, Crb1 localizes just apical to OLM and deficiencies in Crb1 result in OLM defects (Mehalow et al., 2003; van de Pavert et al., 2004). The highest level of Moe labeling was basal to the OLM, as marked by anti-ZO-1, but anti-Moe labeling was also observed apical to the OLM, where panCrb labeling localizes (Fig. 4H,I).

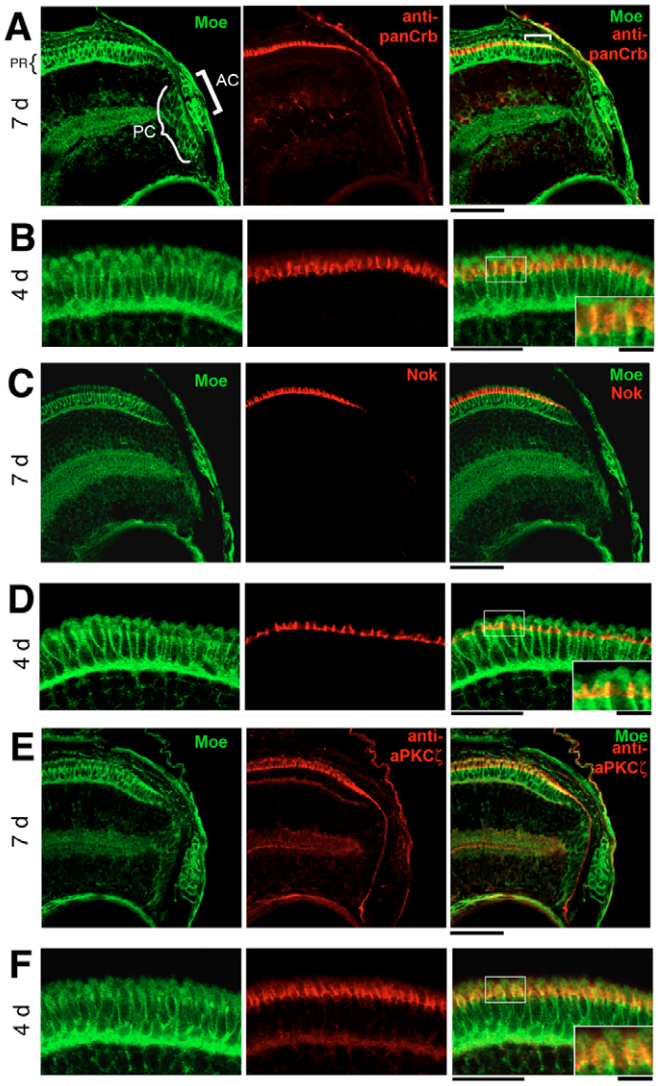

Fig. 3. Localization of Moe, panCrb, Nok and aPKCλ in the retina. (**A**,**B**) Colocalization of guinea pig anti-Moe and panCrb labeling in the peripheral region in 7 dpf eye (A) and photoreceptor layer at 4 dpf (B). AC, anterior chamber; PC, proliferating cells; PR, photoreceptors. (**C**,**D**) Colocalization of guinea pig anti-Moe and anti-Nok labeling in the peripheral region in 7 dpf eye (C) and photoreceptor layer at 4 dpf (D). (**E**,**F**) Colocalization of guinea pig anti-Moe and anti-aPKCζ labeling in the peripheral region in 7 dpf eye (E) and photoreceptor layer at 4 dpf (F). Insets are magnification of boxed areas. Scale bars: 20 μm; 10 μm in insets.

Moe forms a complex with Crumbs proteins, Nok (Pals1) and Has (aPKCλ)

Because Crumbs proteins contain a predicted FERM-binding domain and Moe is a FERM protein, we sought to determine whether Moe and Crumbs proteins physically interact. We found that anti-panCrb (anti-CRB3) antibodies co-immunoprecipitated proteins recognized by anti-Moe (~110 kD), anti-panCrb (~150 kDa) and anti-Nok (~80 kDa) from adult eyes (Fig. 5A). We also found that a fusion protein of Moe that includes the FERM domain (Moe_FERM) pulled down a ~150 kDa protein from larval lysates that was recognized by anti-panCrb antibody (Fig. 5B), further supporting the idea that Moe and Crumbs proteins form a complex. We also showed that Moe_FERM can pull down a ~80 kDa protein recognized by anti-Nok from 3 dpf wild-type larvae and adult eyes but not from *nok* morphants (Fig. 5C,E). Further, we showed by western blot that this ~80 kDa protein was absent in nok^{m520} (Fig. 5D), indicating that this protein is encoded by the *nok* locus.

Given that Moe and aPKCλ colocalized in tissue, we also tested whether aPKCλ (Has) forms a complex with Moe. Moe_FERM pulls down a protein of about 72 kDa that was recognized by antibody to the highly related protein aPKCζ (Fig. 5F). The ~72 kDa protein recognized by anti-aPKCζ was absent in has^{m576} (Fig. 5G), suggesting that this protein is encoded by the *has* locus. A protein of about 52 kDa was also pulled down, but it is unclear whether this represents an isoform of aPKCλ, an aPKCλ degradation product (Coghlan et al., 2000), or another protein that crossreacts with the aPKCζ antibody that was pulled down by Moe_FERM.

Moe interacts directly with Crumbs proteins and Nok (Pals1)

To test whether Moe directly binds to Crumbs proteins, we performed in vitro GST pull-down and far western experiments using purified recombinant proteins. Immobilized His-Moe_FERM interacted with GST-Crb2a^{intra}, GST-Crb2b^{intra} and, to a lesser extent, GST-Crb1^{intra} (Fig. 6A). Furthermore, we showed by far western analysis that GST-Crb1^{intra}, GST-Crb2a^{intra} and GST-Crb2b^{intra} bind to His-Moe_FERM immobilized on nitrocellulose (Fig. 6B). Taken together, our biochemical data suggest that Moe interacts directly with Crumbs proteins.

Both Nok and Pals1 have predicted Band 4.1-binding domains consisting of several lysine residues (Kamberov et al., 2000; Wei and Malicki, 2002) and Stardust has a similar stretch of residues following the SH3 domain. We sought to determine whether Moe, a Band 4.1 protein, interacts directly with Nok (Pals1). We showed that immobilized GST-Nok-Int, containing the predicted Band 4.1-binding motif, pulled down MBP-Moe_FERM but not MBP-Moe C-terminus (Fig. 6C). We also showed in far western experiments that MBP-Moe_FERM bound to nitrocellulose-bound full-length Nok (His-Nok-FL), but not His-Nok-N, which does not contain the predicted Band 4.1-binding domain (Fig. 6D). We also showed that His-Nok-N (including the PDZ domain) directly interacted with

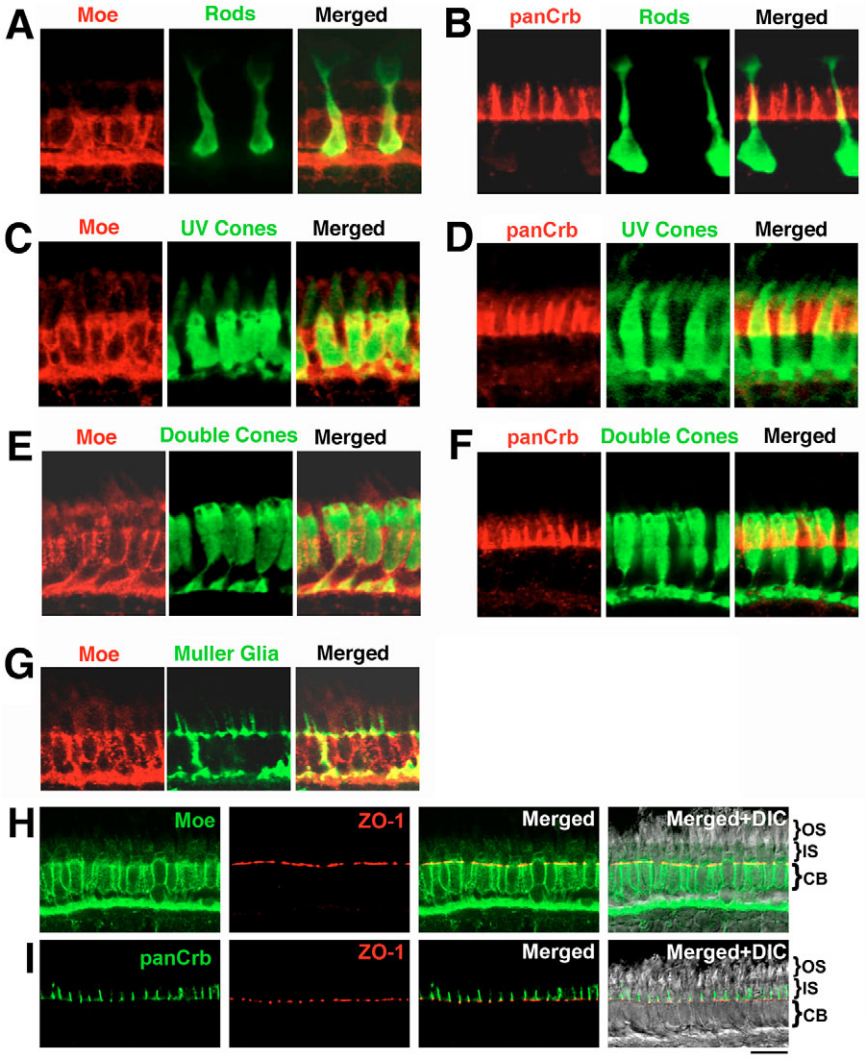

Fig. 4. Localization of Moe and panCrb in specific retinal cell types at 5 dpf. (**A**) Merged guinea pig anti-Moe labeling of rod photoreceptors (in transgenic Xop-GFP fish) with rabbit anti-GFP. (**B**) Merged rabbit anti-panCrb labeling of GFP$^+$ rod photoreceptors with GFP (in transgenic Xop-GFP fish). (**C**) Merged guinea pig anti-Moe labeling of UV cones with GFP (in transgenic UV-GFP fish) labeled with mouse anti-GFP. (**D**) Merged rabbit anti-panCrb labeling of UV cones with GFP (in transgenic UV-GFP fish) labeled with mouse anti-GFP. (**E**) Merged rabbit anti-Moe labeling of double cones with mouse Zpr-1 antibodies. (**F**) Merged rabbit anti-panCrb labeling of double cones with mouse Zpr-1 antibodies. (**G**) Merged guinea pig anti-Moe labeling of Müller glial cells with rabbit anti-Carbonic anhydrase II antibodies. (**H**) Rabbit anti-Moe, mouse anti-ZO-1, merged anti-Moe and anti-ZO-1, and merged with corresponding DIC image. (**I**) Rabbit anti-panCrb, mouse anti-ZO-1, merged anti-Moe and anti-ZO-1, and merged with corresponding DIC image. Scale bar: 10 μm. CB, cell bodies.

both GST-Crb2a^{intra}, GST-Crb2b^{intra} proteins by far western (see Fig. S3 in the supplementary material). Our biochemical analyses show that Moe interacts directly with Crumbs proteins and Nok (Pals1).

The role of Moe in photoreceptor morphogenesis

Given the important role of Crumbs proteins in photoreceptors in *Drosophila*, mice and humans, we sought to determine whether Moe, probably through its interaction with Crumbs proteins, plays a role in vertebrate photoreceptor morphogenesis. Because the position of photoreceptors is so abnormal in *moe* mutants (see Fig. S1O in the supplementary material) (Jensen et al., 2001), examination of their morphology is uninformative. To overcome this limitation, we used genetic mosaic analysis and a transgenic line that expresses GFP in rods (Fadool, 2003). When *moe* mutant cells are transplanted into wild-type hosts at the blastula stage, *moe* mutant cells are almost invariably found in their normal laminar position in the retina (Fig. 7) (Jensen et al., 2004), and so we used this strategy to examine the morphology of GFP$^+$ rods that lack *moe* function. Wild-type rods have a stereotypical morphology: a basal synaptic terminal, a round cell body, a thin apical inner segment (IS) and a thick apical outer segment (OS) filled with rhodopsin (Fig. 7A,B,G,H).

At 6 dpf, the morphology of *moe*$^-$ rods seemed largely normal, but the cells were almost 50% larger than wild-type rods (Fig. 7C-F,O). We measured the accumulated area of the OS versus the IS and cell body and found that the size increase in *moe*$^-$ rods was due largely to an increase in the size of the OS (Fig. 7O). By 10 dpf the morphology of *moe*$^-$ rods was markedly abnormal and the cells were about 50% larger than wild-type rods (Fig. 7I-N,P). Most often *moe*$^-$ rods displayed a coiled apical structure that seemed to encompass both the IS and OS and seemed larger than the combined area of the IS and OS area of wild-type rods (Fig. 7I,K,L-N). We could not accurately measure the OS versus the IS and cell body at 10 dpf because the morphology was so distorted. We grouped transplanted *moe*$^-$ rods into two groups by examining the genotype of neighboring cells; one group included *moe*$^-$ rods that had few *moe*$^-$ neighbors (Fig. 7I-K) and the other group *moe*$^-$ rods had large numbers of *moe*$^-$ neighbors (Figure L-N). Generally, *moe*$^-$ rods with large numbers of *moe*$^-$ neighbors were more abnormal (Fig. 7L-N) than those with mostly wild-type neighbors (Fig. 7I-K). Rhodopsin remained localized to the most distal portion of the cell (Fig. 7I-N), suggesting that apical-basal polarity is preserved. Three-dimensional movies of *moe*$^-$ rods are provided (see movies 1-5 in the supplementary material).

We examined the localization of Crumbs proteins in wild type and *moe*$^-$ rods in our transplant experiments. At 6 and 10 dpf, anti-panCrb labeling in the inner segment in *moe*$^-$ rods did not seem different from wild-type rods (Fig. 7Q-X). Localization of ZO-1

appeared normal around *moe*$^{-}$ rods in genetic mosaics (see Fig. S4 in the supplementary material). The observations that the apical region was expanded in *moe*$^{-}$ rods suggest that Moe may normally act to inhibit apical size in photoreceptors. Interestingly, proper localization of Crumbs proteins to the photoreceptor IS does not appear to require Moe function, contrary to that observed in embryos (see Fig. 2).

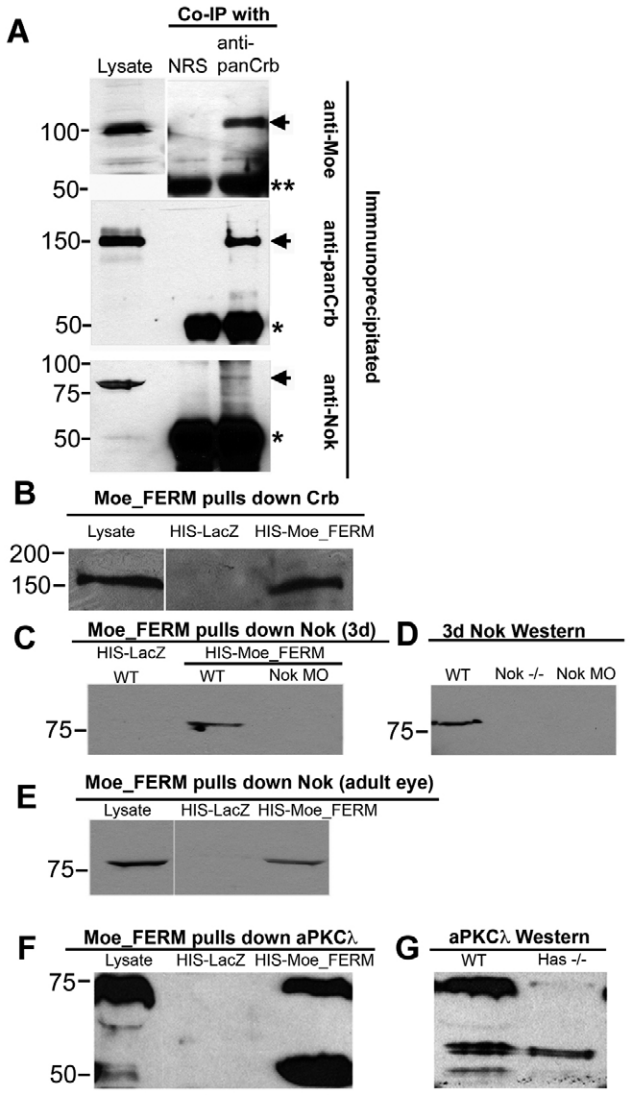

Fig. 5. Moe forms a complex with Crumbs proteins, Nok (Pals1), and Has (aPKCλ). (**A**) Anti-panCrb (anti-CRB3) antibodies co-immunoprecipitate Moe (recognized by guinea pig anti-Moe), Crb2a/b, and Nok from adult eye. NRS, normal rabbit serum. Arrows indicate Moe, Crb2a/b, Nok protein. One asterisk indicates rabbit antibody; two asterisks indicate cross-reactivity of anti-guinea pig-HRP with rabbit antibody. (**B**) His-Moe_FERM, but not His-*LacZ*, pulls down a ~150 kDa protein from 3 dpf larval lysates that is recognized by anti-panCrb antibodies. (**C**) His-Moe_FERM, but not His-*LacZ*, pulls down an ~80 kDa protein from wild-type 3 dpf larval lysates, but not from nok morphant lysates, recognized by anti-Nok antibodies. (**D**) Anti-Nok antibodies recognize an ~80 kDa protein in 3 dpf wild-type lysates that is absent in *nok* mutants and morphants in western blot. (**E**) His-Moe_FERM, but not His-*LacZ*, pulls down an ~80 kDa protein from adult eye lysates recognized by anti-Nok antibodies. (**F**) His-Moe_FERM pulls down ~75 kDa and ~50 kDa proteins from 3 dpf larvae lysates that are recognized by anti-PKCζ antibodies. (**G**) Anti-PKCζ antibodies recognize proteins of approximately 75, 57, 54 and 50 kDa in western blot of 3 dpf wild-type lysates; the 75 kDa and 50 kDa proteins are absent in lysates from *has* mutants (Has$^{-/-}$).

DISCUSSION

The present study shows that Moe, a FERM protein, colocalizes with vertebrate Crumbs orthologs, co-immunoprecipitates with Crb2a, can directly interact with three zebrafish Crumbs proteins (Crb1, Crb2a and Crb2b) and is required for embryonic localization of Crumbs proteins. The similarity between the embryonic phenotypes of *moe* mutants and *crb2a* morphants suggests that Moe is a crucial regulator of *crb2a* function. We further show that Moe negatively regulates the size of the apical domain in rod photoreceptors. We propose that Moe, through its interactions with Crumbs proteins, has three independent roles in central nervous system development: brain ventricle formation, retinal lamination and photoreceptor morphogenesis. The role of Moe in brain ventricle formation may be to localize proteins that promote inflation of the ventricles by regulating ion transport and fluid dynamics. Consistent with this potential role is our observation that the peptide in the FERM domain of Band 4.1 that binds an anion exchanger (Jons and Drenckhahn, 1992) is highly conserved in Moe. It seems that ion exchangers play a crucial role in brain ventricle formation, as mutations in a *Na+K+ATPase* cause failure of brain ventricle formation that is very similar to the *moe* and *nok* mutations (Yuan and Joseph, 2004; Lowery and Sive, 2005). Localization of such proteins in the retinal epithelium would be disruptive, because formation of a lumen would separate the retinal epithelium from the retinal pigmented epithelium. We still do not fully understand why *moe* loss of function has such a dramatic effect on retinal epithelial integrity while the integrity of the brain epithelium seems relatively normal even though *moe* and *crb2a* are expressed in both tissues. Protein function redundancy seems unlikely to account for the differences in retina brain defects, as we detected no expression of the paralog of *moe*, *ehm2*, in the brain (A.M.J. and Y.-C.H., unpublished), and panCrb labeling, which we showed recognizes all zebrafish Crumbs proteins, is completely lost in the brain of *ome* (*crb2a*) mutants.

The most remarkable observation made is that the apical membrane in rods is expanded by *moe* loss of function, and *moe*$^{-}$ rods are almost 50% larger than wild-type rods. The morphology of *moe*$^{-}$ photoreceptors was largely normal at 6 dpf, which is 3 days after the onset of rod IS and OS formation (Schmitt and Dowling, 1999), but by 10 dpf, most rods exhibited an abnormal morphology that included a distinctive coiled shape. By morphology we were unable to always distinguish the IS from the OS at 10 dpf, but Rhodopsin remained localized distally, suggesting that apical/basal polarity is preserved. The conspicuous coiled morphology of the rods could be intrinsic, caused by adhesion defects or due to space constraints imposed by neighboring cells.

The vertebrate photoreceptor OS is a highly modified cilium (Röhlich, 1975). Vertebrate Crumbs proteins have been shown to be important for ciliogenesis; siRNA knockdown of *crb3* leads to a dramatic reduction in the number of ciliated MDCK cells (Fan et al., 2004), and in zebrafish inhibition of *crb3a* shortens auditory kinocilia, and morpholino knockdown of *crb2b* shortens nephric cilia and also shortens photoreceptor ISs, which lie below the OS (Omori and Malicki, 2006). Mice with a *Crb1* mutation have shortened ISs and OSs (Mehalow et al., 2003). The *Drosophila* photoreceptor stalk region, which may be a homologous structure to the IS in vertebrate photoreceptors, is shortened by *crb* loss of function and is expanded by overexpression of full-length *crb*

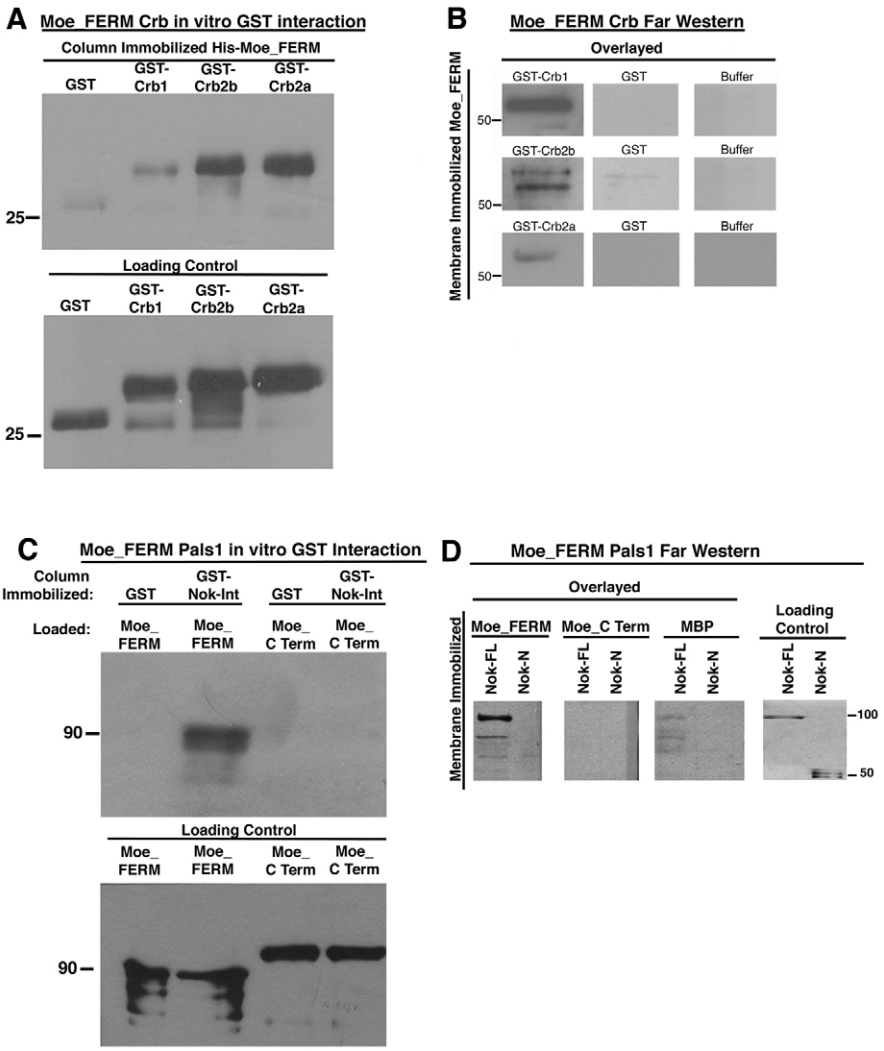

Fig. 6. Moe directly binds Crumbs proteins and Nok (Pals1). (**A**) His-Moe_FERM binds GST-Crb1, GST-Crb2a and GST-Crb2b. The blot was probed with anti-GST antibodies. Loading control, anti-HIS western blot of one-fortieth the material used in the experiment. (**B**) Far western analysis shows that membrane immobilized His-Moe_FERM (molecular weight approximately 55 kDa) binds GST-Crb1, GST-Crb2a and GST-Crb2b, but not GST alone, and no GST immunoreactivity is observed when His-Moe_FERM is incubated with buffer alone. Far westerns were probed with anti-GST antibodies. (**C**) GST-Nok-Int binds MBP-Moe_FERM but not MBP-Moe_C Term. The blot was probed with anti-MBP. Loading control, anti-GST western blot of one-twentieth the amount of MBP proteins loaded. (**D**) MBP-Moe_FERM but not MBP-Moe_C Term binds to membrane immobilized His-Nok_FL (Full Length) but not to His-Nok_N-term, which does not contain the predicted Band 4.1-binding domain. Far Westerns probed with anti-MBP. Loading control, anti-HIS Western blot of one-tenth the amount of His-fusion proteins.

(Izaddoost et al., 2002; Pellikka et al., 2002). Overexpression of *crumbs* also expands the apical domain of ectodermal epithelia in the *Drosophila* embryo (Wodarz et al., 1995). Our observations that OSs are expanded in *moe*$^{-}$ rods, taken with those above, suggest that Moe may be a negative regulator of Crumbs protein function in photoreceptors. We did not observe a significant increase in IS size at 6 dpf, and at 10 dpf we were unable to confidently identify the different compartments (cell body, IS and OS) in *moe*$^{-}$ rods, so it remains to be determined whether the IS is affected by loss of *moe* function. *Drosophila* Yurt (Moe ortholog) also appears to act as negative regulator of apical membrane size and is shown to interact directly with Crumbs (Laprise et al., 2006). Collectively, our observations and those of others lead us to propose that Crb proteins are good candidates to be part of the molecular mechanism that regulates daily apical renewal in photoreceptors and that Moe may be an important negative regulator of this mechanism.

Photoreceptors, both in invertebrates and vertebrates, periodically shed distal apical membrane and then renew to replace the shed material. It has been estimated that as much as 10% of the OS is shed and renewed daily in mammals (Young, 1967), and both processes are regulated by cyclic light (Besharse et al., 1977; Hollyfield and Rayborn, 1979; Stowe, 1980); experiments in the locust suggest these processes are controlled locally (Williams, 1982). To our knowledge, no molecules or molecular mechanisms have been identified that regulate the process of apical membrane renewal in photoreceptors, although many genes, including that encoding Rhodopsin, are required for the formation of the OS (Lem et al., 1999). A prediction of this idea is that Crb1/Crb2 complex should be regulated by light. This prediction is supported already by the observation that exposure to bright light accelerates photoreceptor death in the mouse *crb1* knockout (van de Pavert et al., 2004) and *Drosophila crb* mutant retina (Johnson et al., 2002). The potential role of Crb1/Crb2 and its regulation by Moe has important implications in the etiology of photoreceptor degeneration diseases, which often are marked first by a shortening of the IS and OS.

Levels of Crumbs proteins may be especially critical for photoreceptors. Mouse and zebrafish photoreceptors express two *crumbs* genes (Fig. 1) (den Hollander et al., 2002; van den Hurk et al., 2005), and in *Drosophila* the stalk is slightly shorter in *crb*$^{-/+}$ photoreceptors (Pellikka et al., 2002). Perhaps the differences between *Crb1* loss of function in humans and mice reflect differences in the compensatory function of *Crb2*. Although *crb1* is not expressed in the zebrafish retina (data not shown) (Omori and Malicki, 2006), photoreceptors still express two *crumbs* genes, *crb2a* and *crb2b*; perhaps one of these *crb2* genes may have adopted the function of *crb1* in mammals.

We showed that Moe and panCrb localization at the brain and retina ventricular surface depend on reciprocal Moe/Crb protein function and Nok function. The intracellular punctate panCrb labeling in the cell bodies of the wild-type brain and retinal

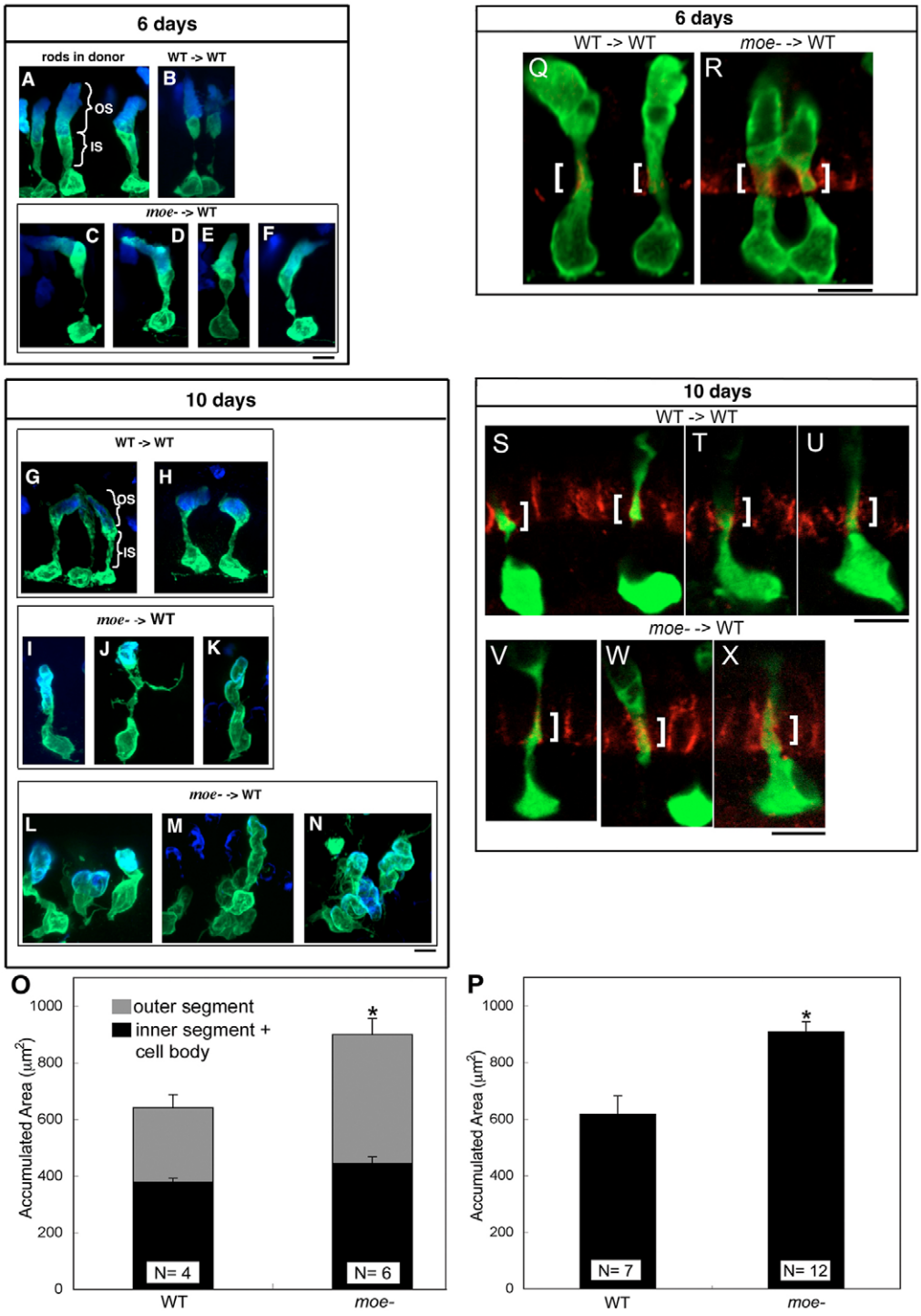

Fig. 7. Moe is required for normal photoreceptor morphology and negatively regulates apical size. (**A**,**B**) Wild-type GFP$^+$ rods labeled with anti-Rhodopsin antibody (blue) in a wild-type donor retina (A) and wild-type GFP$^+$ rods transplanted into a wild-type host (B) at 6 dpf. (**C-F**) *moe*$^-$ GFP$^+$ rods transplanted into wild-type hosts at 6 dpf. (**G**,**H**) Wild-type GFP$^+$ rods transplanted into wild-type hosts at 10 dpf. (**I-K**) *moe*$^-$ GFP$^+$ rods transplanted into wild-type hosts with few *moe*$^-$ neighbors at 10 dpf. (**L-N**) *moe*$^-$ GFP$^+$ rods transplanted into wild-type hosts with large numbers of *moe*$^-$ neighbors at 10 dpf. (L) Three rods, (M) two to three intermingled rods and (N) three intermingled rods. (**O**) At 6 dpf, the accumulated area *moe*$^-$ rods is larger than wild-type rods. The increase in size is accounted for largely by an increase in outer segments whereas there is no significanct difference in the inner segments plus cell body (error bars shown; s.e.m.; *, P=0.04). The total accumulated area of *moe*$^-$ rods (901+/–64) is significantly greater than wild-type rods (641+/–37) (P=0.015). Numbers of individuals (N) included. (**P**) At 10 dpf, the accumulated area of *moe*$^-$ rods is larger than wild-type rods. (*, P=0.001; Student's t-test). (Q-X) panCrb labeling (red) in a single optical section (0.38 μm) of wild-type (**Q**) and *moe*$^-$ (**R**) GFP$^+$ rods at 6 dpf and wild-type (**S-U**) and *moe*$^-$ GFP$^+$ rods (**V-X**) at 10 dpf. White brackets indicate the region of panCrb localization in the inner segment. Scale bars: 5 μm.

neuroepithelium is reduced or absent in *moe* mutants (Fig. 2D,F), but overall protein levels are unaffected, suggesting that Moe may be required for the intracellular trafficking of Crb protein through organelles. Interestingly, disruption of Crb trafficking through the endosomal pathway leads to an upregulation of cell-surface Crb protein (Lu and Bilder, 2005), and recently two other FERM proteins, Merlin and Expanded, have been implicated in regulating cell-surface receptor localization, abundance and turnover (Maitra et al., 2006). The loss of apical panCrb labeling in *moe*$^-$ embryos contrasts with the normal Crumbs protein localization observed in *moe*$^-$ rods, suggesting that additional proteins or cellular or molecular mechanisms operate to localize Crumbs proteins in photoreceptors. Crumbs-expressing wild-type Müller glia, which send processes into the IS region, may help to localize Crumbs protein in *moe*$^-$ rods.

We also show that Moe forms a complex with Nok (Pals1) and Has (aPKCλ) and that Moe can interact directly with Nok. The interaction between Moe and Nok may serve to regulate the interaction between Nok and Crumbs proteins or to bring Nok into the Crumbs complex. The former hypothesis is supported by studies of the Glycophorin C (GPC) ternary complex, which includes the Maguk protein, p55, and the FERM protein, Band 4.1, showing that

the inclusion of Band 4.1 in the complex increases the affinity of p55 for GPC by an order of magnitude (Nunomura et al., 2000). The interaction of Moe with aPKCλ may be mediated by the Par3/6 complex (Wodarz et al., 2000; Hurd et al., 2003; Lemmers et al., 2004; Nam and Choi, 2003); perhaps aPKCλ regulates the interaction between Moe and Crumbs proteins, as DaPKC phosphorylates *Drosophila* Crumbs in the FERM-binding domain and phosphorylation of Crb is required for apical localization of Crb in *Drosophila* embryos (Sotillos et al., 2004). In addition, Moe itself may be a target for aPKCλ regulation, as there are several potential serine and threonine phosphorylation sites (A.M.J., unpublished).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/24/4849/DC1

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